

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

37

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|-----------|--|
| (51) International Patent Classification ⁶: A61K 39/395, C07K 16/30, C12N 5/20, 5/24, C12P 21/08 | A1 | (11) International Publication Number: WO 96/00084 (43) International Publication Date: 4 January 1996 (04.01.96) |
| (21) International Application Number: PCT/US95/07928 (22) International Filing Date: 21 June 1995 (21.06.95) (30) Priority Data: 08/265,411 24 June 1994 (24.06.94) US (71)(72) Applicants and Inventors: TORCHILIN, Vladimir, P. [RU/US]; 12 Shipway Place, Charlestown, MA 02129 (US). IAKOUBOV, Leonid, Z. [RU/US]; 51 Parker Terrace, Newton, MA 02159 (US). (74) Agent: CLARK, Paul, T.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US). | | (81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> |
| (54) Title: USE OF AUTOANTIBODIES FOR TUMOR THERAPY AND PROPHYLAXIS (57) Abstract A method of inhibiting the growth of a tumor cell in a mammal by administering to the mammal an autoantibody, e.g., an antinuclear autoantibody from an aged mammal, that binds to either one or both of a surface of a tumor cell and a protein released from a dead tumor cell. Also disclosed are natural and monoclonal antinuclear autoantibodies from aged mammals and a hybridoma cell line producing a monoclonal antinuclear autoantibody. | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|--|----|--------------------------|
| AT | Austria | GB | United Kingdom | MR | Mauritania |
| AU | Australia | GE | Georgia | MW | Malawi |
| BB | Barbados | GN | Guinea | NE | Niger |
| BE | Belgium | GR | Greece | NL | Netherlands |
| BF | Burkina Faso | HU | Hungary | NO | Norway |
| BG | Bulgaria | IE | Ireland | NZ | New Zealand |
| BJ | Benin | IT | Italy | PL | Poland |
| BR | Brazil | JP | Japan | PT | Portugal |
| BY | Belarus | KE | Kenya | RO | Romania |
| CA | Canada | KG | Kyrgyzstan | RU | Russian Federation |
| CF | Central African Republic | KP | Democratic People's Republic of Korea | SD | Sudan |
| CG | Congo | KR | Republic of Korea | SE | Sweden |
| CH | Switzerland | KZ | Kazakhstan | SI | Slovenia |
| CI | Côte d'Ivoire | LI | Liechtenstein | SK | Slovakia |
| CM | Cameroon | LK | Sri Lanka | SN | Senegal |
| CN | China | LU | Luxembourg | TD | Chad |
| CS | Czechoslovakia | LV | Latvia | TG | Togo |
| CZ | Czech Republic | MC | Monaco | TJ | Tajikistan |
| DE | Germany | MD | Republic of Moldova | TT | Trinidad and Tobago |
| DK | Denmark | MG | Madagascar | UA | Ukraine |
| ES | Spain | ML | Mali | US | United States of America |
| FI | Finland | MN | Mongolia | UZ | Uzbekistan |
| FR | France | | | VN | Viet Nam |
| GA | Gabon | | | | |

- 1 -

USE OF AUTOANTIBODIES FOR
TUMOR THERAPY AND PROPHYLAXIS

Background of the Invention

5 The invention relates to the use of autoantibodies for tumor therapy and prophylaxis.

 Neoplasia is often caused by somatic mutations. When these mutations accumulate with age, the burden on the immune system, which controls neoplasia as one of its
10 functions, is increased. However, despite increased cancer-related mortality rates in the aged, many aged individuals do not develop tumors. One explanation for this immunity is that certain natural antibodies play a role in humoral anti-tumor immunity (Chow et al., Int. J.
15 Cancer, 27:459-469, 1981). However, little is known about the antigenic specificity of such antibodies.

 One type of natural antibody found in the immune system is the autoantibody. These autoantibodies have been repeatedly found in significantly higher titers in
20 older humans and laboratory animals without overt disease than in younger controls (Whitaker, et al., Clin. Res., 14:143, 1966; Cammarata, et al., JAMA, 199:115-118, 1967; Siegel, et al., Immunology, 22:457-463, 1972). Hybridomas that produce natural autoantibodies from mice
25 of different ages have been described (Sakharova, et al., Zh. Obshch. Biol., 47:625-630, 1986).

 The occurrence of autoantibodies in the aged is believed to serve as a marker of a disregulated immune system, and these antibodies recently have been
30 prescribed with tumor growth-enhancing properties (Ben-Yehuda, et al., Cancer Invest., 10:525-531, 1992). In earlier studies, others have noted that elevated levels of serum autoantibodies can result in the development of autoimmune systemic diseases (Dixon, et al., Progress in
35 Immunology, 959-995, New York: Academic Press, 1980), and that antinuclear autoantibody suppression by the

- 2 -

immunosuppressive drug cyclophosphamide may correlate with growth of a proliferating lymphoreticular neoplasm (Walker, et al., Clin. Exp. Immunol., 24:210-217, 1976). Hahn et al., Arthrit. Rheumat., 18:145-152 (1975),

- 5 concluded that cyclophosphamide enhanced production of neoplasms such as lymphoreticular neoplasms, carcinomas, and sarcomas.

Summary of the Invention

- The invention is based on the discovery that
- 10 natural antinuclear autoantibodies (ANA), e.g., from aged individuals, can be used to treat neoplasia, both existing tumors, and as a prophylactic measure. This protective effect was demonstrated for a natural monoclonal ANA (2C5) from an aged BALB/c mouse that
- 15 dramatically inhibited the development of aggressive syngeneic EL4 T cell lymphoma in young mice, whereas control antibodies of the same isotype were without any effect.

- The antibodies of the invention are characterized
- 20 in that they (1) are autoantibodies, (2) bind to the surface of living tumor cells, e.g., EL4 T lymphoma cells, but not to normal T cells, (3) are antinuclear autoantibodies, i.e., bind to an antigen found in cell nuclei, e.g., a deoxyribonucleoprotein (DNP), and/or (4)
- 25 bind to a protein, e.g., a DNP, released from dead tumor cells. The autoantibodies are found in higher titers in aged mammals, but can exist in young mammals as well.

- In general, the invention features a composition for use in inhibiting the growth of a tumor cell in a
- 30 mammal, the composition including an autoantibody that binds specifically to (i) a surface of a living tumor cell, e.g., EL4, VERO neoplastic epithelial cells from green monkey kidney, a human lymphoma cell, a human myeloma cell, a human sarcoma cell, or the like, and (ii)

- 3 -

a protein released from a dead tumor cell, e.g., DNP, or an antigen in the nucleus of said tumor cell, and (iii) does not bind to the surface of normal T cells.

The invention also features a method of inhibiting the growth, e.g., proliferation or actual increase in size, of a tumor cell in a mammal, e.g., a human, by administering to the mammal an autoantibody-containing composition of the invention. In addition, the invention features the use of the autoantibody-containing composition of the invention for the manufacture of a medicament for inhibiting the growth of a tumor cell in a mammal.

The autoantibody can be a substantially pure autoantibody isolated from serum from an aged mammal, such as a human or a murine mammal, or a monoclonal antibody produced by a hybridoma of a cell from an aged mammal.

The composition or medicament can be used to treat various human tumor cells including a human sarcoma, myeloma, carcinoma, or lymphoma cell, and can be administered before a tumor cell is detected in the mammal, e.g., prophylactically to prevent the growth of tumor cells appearing in the mammal following administration of the autoantibody, or after a tumor cell is detected in the mammal, to prevent the further growth of tumor cells existing in the mammal prior to administration of the autoantibody.

The invention also features a method of determining whether an autoantibody is capable of inhibiting growth of a tumor cell, e.g., EL4, VERO neoplastic epithelial cell from green monkey kidney, a human lymphoma cell, a human myeloma cell, a human cell, a human sarcoma cell, or the like, by (a) obtaining a candidate autoantibody; (b) testing binding of the autoantibody to the surface of a tumor cell; (c) testing

- 4 -

binding of the autoantibody to the surface of a normal T cell; and (d) testing binding of the autoantibody to a protein released from a dead tumor cell, e.g., a DNP, or an antigen in the nucleus of said tumor cell, wherein the
5 autoantibody is capable of inhibiting growth of a tumor cell when the autoantibody tests positive in both of steps (b) and (d), and tests negative in step (c).

The invention further features a composition for inhibiting the growth of a tumor cell in a mammal, e.g.,
10 a human, that includes an autoantibody linked, e.g., covalently or non-covalently bound, to a bioactive agent such as a cytotoxic agent or a cytokine. Cytotoxic agents include but are not limited to: saponin, ricin, ricin A-chain, abrin, abrin A-chain, diphtheria toxin,
15 diphtheria toxin A-chain, exotoxin A-chain, daunomycin, daunorubicin, doxorubicin, mitomycin C, 5-fluorouracil, cytosine arabinoside, colchicine, cytochalasin B, bleomycin, vincristine, vinblastine, and methotrexate. Cytokines include, but are not limited to: interleukins,
20 e.g., IL-2, growth factors, e.g., GM-CSF, and interferons, e.g., gamma interferon.

The invention also features a hybridoma cell which produces an autoantibody that inhibits tumor cell growth in a mammal. The autoantibody of the invention can be a
25 polyclonal ANA from an aged mammal or a monoclonal antibody, e.g., antinuclear autoantibody 2C5, produced by a hybridoma cell, e.g., a hybridoma cell from the hybridoma cell line having the A.T.C.C. accession number CRL 11667. The autoantibody of the invention can be a
30 substantially pure antibody.

By "substantially pure" is meant that the antibody provided by the invention is least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated.
35 Preferably, the preparation is at least 75%, more

- 5 -

preferably at least 90%, and most preferably at least 99%, by weight, autoantibody. A substantially pure antibody may be obtained, for example, by extraction from a natural source such as mammalian, e.g., human, serum; 5 preparation of a hybridoma cell producing the antibody, by expression of a recombinant nucleic acid encoding a monoclonal antibody, or by chemically synthesizing the protein. Purity can be measured by any appropriate standard method, e.g., column chromatography, 10 polyacrylamide gel electrophoresis, or HPLC analysis.

As used herein, the phrase "autoantibody from an aged mammal" means a natural autoantibody taken directly from a mammal that is old, i.e., one that has lived 60% or more of the life span expected for that species. This 15 phrase also encompasses autoantibodies produced by a cell taken from an old mammal or progeny of that cell, for example, by generation of hybridoma cells.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as 20 commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are 25 described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the detailed description, and from 30 the claims.

Brief Description of the Drawings

Figs. 1A and 1B are photographs of five mice showing suppression of tumor growth in the presence of monoclonal ANA 2C5 and extensive tumor growth in the

- 6 -

presence of an isotype matched control antibody, respectively.

Figs. 2A and 2B are graphs showing flow cytometric analysis of monoclonal ANA 2C5 binding to EL4 T cell lymphoma cells from an *in vitro* culture, and lack of binding to thymocytes from 5 week old mice, respectively.

Fig. 3 is a graph showing flow cytometric analysis of monoclonal ANA 2C5 binding to S49 T cell lymphoma cells from an *in vitro* culture.

10 Detailed Description

Applicants have discovered that natural autoantibodies, such as ANA from aged individuals, can be used to treat existing tumors and as a prophylactic measure to prevent tumors or their metastases. There now follows a description of the isolation and characterization of ANA from an aged mouse, and the production and testing of monoclonal ANA from mice for use in the inhibition of tumor cell growth in a mammal. These examples are provided for the purpose of illustrating the invention, and should not be construed as limiting. Moreover, the techniques can be applied generally to the isolation, characterization, and use of autoantibodies from human serum, and the production of human monoclonal autoantibodies.

25 Autoantibodies

The autoantibodies, e.g., ANA, can be from any mammalian species, including, murine, human or the like, or combinations thereof, such as chimeric antibodies, having a human constant region and a mouse or other mammalian source variable region. The important characteristic is that the autoantibody binds specifically to the surface of living tumor cells, e.g., EL4 T lymphoma cells, but not to normal T cells, or binds

- 7 -

to a protein, e.g., deoxyribonucleoprotein, released from the dead tumor cells.

Detection of ANA by Indirect Immunofluorescent

Test

5 ANA can be detected in the serum of mammals, e.g., human patients, using an indirect immunofluorescence procedure that involves the use of mammalian liver substrate, e.g., rat liver or human liver cell line HEP2. In this procedure, human serum is placed on a cryostat
10 section of rat liver that permits attachment of immunoglobulin molecules with antinuclear specificity. The slide with the rat liver sections is washed with phosphate-buffered saline (PBS) and the fluorescein-conjugated goat anti-human IgG (Antibodies Inc.) is
15 added. After washing again with PBS, the fluorescein-conjugated goat anti-human IgG is visualized by fluorescence microscopy. Serum samples are diluted with PBS; the highest titer that produces a positive test for ANA is used as the ANA titer.

20 The pattern of immunofluorescence often corresponds to a particular antigen against which the antibodies are directed. For example, a homogeneous pattern of immunofluorescence in the cell nucleus suggests the presence of antibodies directed against DNA
25 or certain DNA binding proteins such as histones. A speckled pattern is characteristic of antibodies directed against nuclear ribonucleoproteins (nRNP) such as Sm, Ro, and La antigens.

Preparation of Antibodies

30 Once detected and isolated, autoantibodies, e.g., ANA, can be obtained as monoclonal autoantibodies by hybridoma formation and expression in the hybridoma supernatant culture, or produced by ascites. The antibody can also be a monoclonal antibody fragment, such

- 8 -

as FAB, F(ab₂), Fv, a recombinant variable region, or the like.

ANA from an aged mammal are prepared by pooling sera of an immune mammal or mammals, e.g., a mouse or a human, followed by fractionation of the IgG component from the plasma or sera. Human or mouse monoclonal antibody producing cell lines can be prepared by standard transformation and hybridoma technology (see, e.g., Methods in Enzymology, Vol. 121, Sections I and II (1986) eds. J.J. Langone and H.V. Vunakis, Academic Press).

Monoclonal natural ANA 2C5 (2C5) from non-immunized healthy, aged mice was obtained and characterized as belonging to the subclass IgG2a (Sakharova, et al., Zh. Obshch. Biol., 47:625-630, 1986). 2C5 was obtained as follows. Splenocytes from two non-immunized healthy aged (26 months old out of an average life span of up to 30 months) female Balb/c mice were fused with murine myeloma cell line P3X63-Ag8.653 (ATCC No. CRL1580). Several hundred resulting hybridoma clones were tested for autoreactivity in a standard radioimmunoassay with homogenates of different tissues (liver, kidney, brain). Homogenates of these organs were incubated with the supernatants of the hybridoma clones, washed three times in phosphate-buffered saline with 3% bovine sera albumin (PBS-3), incubated with 125-I-labeled anti-mouse antibodies in PBS-3, washed three times in PBS-3 and analyzed in a γ -counter. About 5% of hybridomas obtained were positive in this test, and were positive with all three targets. Thus, these antibodies are not tissue-specific, and are clearly autoantibodies.

Further characterization of the target of 2C5 was achieved in a test of indirect immunostaining of mouse or rat brain slices. Cryostat slices were fixed in acetone, washed 3 x 5 minutes in PBS-3, incubated for 20 minutes with hybridoma supernatants, washed, and incubated with

- 9 -

h rseradish peroxidase-labelled anti-mouse antibodies. Color reaction was developed with 3,4 diaminobenzidine in a standard procedure.

All the hybridomas found to be positive in the
5 radioimmunoassay with homogenates were also positive in an immunocytochemistry study of mouse or rat brain slices, showing reactivity with the nuclei of all the cells. In contrast, no antinuclear reactivity was seen with homogenates-positive hybridomas obtained in a
10 similar procedure from newborn (4-5 days old) or adult (6 months old) non-immunized healthy mice. Homogeneous, but not speckled, patterns of nuclear staining indicated that the antibody's target is DNA or some DNA-bound protein. Thus, the autoantibody was also an antinuclear
15 autoantibody (ANA).

Hybridoma 2C5 was chosen for further analysis because it showed intensive nuclear reactivity and high reactivity with the surface of transformed tumor cell lines from *in vitro* or *ex vivo* cultures such as EL4 mouse
20 thymoma, S49 mouse T lymphoma, P3X63-Ag8.653 mouse myeloma, and a VERO neoplastic epithelial cell line from green monkey kidney. The hybridoma producing this monoclonal antibody was deposited on June 22, 1994 with the A.T.C.C. in Rockville, Maryland, under the Accession
25 No. CRL 11667.

Monoclonal antibody 2C5 and the isotype matched control antibody, UPC10, (Cappel, Durham, NC) were purified from ascites fluid by precipitation with 40% ammonium sulfate, or further purified using ion-exchange
30 chromatography on CM-agarose (Pharmacia Biotech, Inc., Piscataway, NJ) using standard techniques well known to those skilled in the art (see, e.g., Jiskoot et al., "Two-Step Purification of a Murine Monoclonal Antibody Intended for Therapeutic Application in Man", J. Immunol.
35 Methods, 124:143-156, 1989; Ausubel et al., Current

- 10 -

Protocols in Molecular Biology, John Wiley & Sons, New York, 1989; and Fisher, *Laboratory Techniques in Biochemistry and Molecular Biology*, eds., Work and Burdon, Elsevier, 1980).

- 5 The antibody purity was characterized with HPLC using standard techniques. Both ammonium sulfate-precipitated and ammonium sulfate/CM-agarose-purified preparations gave similar results in *in vivo* experiments.

Humanizing Antibodies

- 10 Since, for the most part, monoclonal antibodies are produced in species other than humans, they are often immunogenic to humans. In order to successfully use these monoclonal antibodies in the treatment of humans, it may be necessary to create a chimeric antibody
- 15 molecule wherein the portion of the polypeptide involved with ligand binding (the variable region) is derived from one species, and the portion involved with providing structural stability and other biological functions (the constant region) is derived from a human antibody.
- 20 Methods for producing chimeric antibodies in which the variable domain is derived from one host and the constant domain is derived from a second host are well known to those skilled in the art. See, for example, Neuberger et al., WO Publication No. 86/01533, priority 9/3/84;
- 25 Morrison et al., EP Publication No. 0 173 494, priority 8/27/84.

- An alternative method, in which an antibody is produced by replacing only the complementarily determining regions (CDRs) of the variable region with
- 30 the CDRs from an immunoglobulin of the desired antigenic specificity, is described by Winter (GB Publication No. 2 188 638, priority 3/27/86). Murine monoclonals can be made compatible with human therapeutic use by producing an antibody containing a human Fc portion (Morrison, S.L.
- 35 (1985) Science 229:1202-1207). Established procedures

- 11 -

allow construction, expression, and purification of such a hybrid monoclonal antibody. Regimens for administering immune globulin therapeutically have previously been used for a number of infectious diseases.

5 In addition, antibodies can be generated by non-human mammalian hosts that contain xenogeneic human DNA segments encoding human Ig loci for response to immunogens according to the protocol in Kuncherlapati et al., WO 94/02602.

10 As used herein, the term "antibody" is meant to encompass monoclonal or polyclonal antibodies, whole, intact antibodies or antibody fragments having the immunological activity of the whole antibody. Also encompassed within the term "antibody" are chimeric
15 antibodies having the variable and constant from different host species, or those wherein only the CDRs are replaced.

Human Monoclonal Antibodies

Some human ANA give a homogeneous pattern of
20 nuclear staining and react with cell plasma membranes, similar to 2C5 (Rekvig, Scand. J. Immunol., 29:7-13, 1989). Thus, hybridomas of completely human origin that produce human autoantibodies, e.g., ANA, can be obtained by fusing B cells (e.g., peripheral blood B cells) from
25 an ANA-positive healthy, aged individual with a human "fusion partner" cell line such as UC 729-6 or SHFP-1 as described in McKnight et al., Human Antibodies and Hybridomas, 1:77-82 (1990).

Testing Anti-Tumor Autoantibodies

30 Anti-tumor autoantibodies that fall within the scope of the present invention can be characterized using the following standard assays. First, they must be autoantibodies, which can be determined using standard tests for autoantibodies, e.g., ELISA with standard

- 12 -

autoantigens. Second, they must bind to the surface of a tumor cell and/or a protein released by dead tumor cells. These two binding characteristics can be determined using standard flow cytometry techniques and an ELISA assay, 5 respectively, as described below.

Assay for Tumor Cell Surface Binding

The binding of a monoclonal ANA (e.g., 2C5 described above, or a monoclonal ANA from an aged mammal such as a human) to tumor cells (e.g., lymphoma or other 10 tumor cell, where the tumor cell is from a mouse or a human) was monitored by flow cytometry using standard techniques. This assay can be used to determine whether a given ANA binds to a tumor cell surface antigen, and to determine whether a given tumor cell can be treated with 15 a selected ANA.

Cell viability at the start of the procedure was more than 95% as determined by a standard Trypan Blue exclusion test.

Cells in single cell suspensions (EL4 T lymphoma, 20 S49 T lymphoma, and P3X63-Ag8.653 myeloma) or in monolayers (for non-suspension cells such as VERO cells (green monkey kidney epithelium tumor) were incubated for 20 minutes with monoclonal antibody 2C5 or isotype matched control myeloma antibody UPC10 (5-10 μ g/ml in 25 culture media with 10% bovine calf serum (CM10, HyClone, Logan, UT), washed twice in HBSS (Cellgrow, Herndon, VA), incubated 20 minutes with FITC-labelled F(ab)2 fragments of goat anti-mouse antibodies (Cappel) (diluted 1:100 with CM10), again washed twice with HBSS, and fixed in a 30 4% paraformaldehyde solution in PBS and studied using a fluorescent microscope or flow cytometer. All the procedures were carried out at 20°C.

Cells were analyzed using a FACSCAN analyzer (Becton Dickinson and Co., Bedford, MA), and were life 35 gated using forward and side scatter to exclude debris

- 13 -

and d ad cells according to manufacturers instructions. Life gating is a standard hardware or software program that allows analysis of only living cells. The reaction with the surface of attached VERO cells was examined only
5 with a fluorescent microscope. A trypan blue exclusion test was used to exclude the reactivity due to any interaction with intracellular antigens. The reaction of 2C5 with cell surface antigens of suspension cells EL4 thymoma, S49 T lymphoma, and P3/Ag8.653 myeloma, was
10 demonstrated both with the microscope and with the flow cytometer.

As shown in Fig. 2A, monoclonal antibody 2C5 bound to EL4 T cell lymphoma from an *in vitro* culture, but did not bind to thymocytes from an intact 5 weeks old C57BL/6
15 mouse (Fig. 2B). In these two graphs, the relative fluorescence of FITC-labeled anti-mouse F(ab)2 fragments alone is shown as a solid line, of control antibody UPC10 plus FITC-labeled anti-mouse F(ab)2 fragments is shown as a widely dotted line, and of the 2C5 plus FITC-labeled
20 anti-mouse F(ab)2 fragments is shown as a closely dotted line. Thus, Fig. 2A shows increased relative fluorescence only for the closely dotted line, and Fig. 2B shows no increased relative fluorescence.

As shown in Fig. 3, 2C5 also bound to S49 T
25 lymphoma (closely dotted line) whereas control antibody UPC10 did not. Antibody 2C5 also bound to P3/Ag8.653 myeloma and green monkey kidney epithelium tumor cells. Based on these results, 2C5 should also bind to other tumor cells, including human tumor cells.

30 ELISA Assay

A standard ELISA assay can be used to determine whether a given autoantibody binds to nuclear antigens and/or proteins released from dead cancer cells. Nuclear antigens for anti-tumor ANA such as 2C5 include histones,
35 which play a structural role in chromatin organization,

- 14 -

and non-histone proteins regulating DNA transcription and replication such as La- and Ku-antigens.

The interaction of monoclonal antibody 2C5 with various nuclear autoantigens was tested with 96-well plates coated with the nuclear autoantigens listed in Table 1 below obtained from Diamedix Corp., Miami, FL. The coating of ELISA plates with autoantigens released from the dead EL4 lymphoma cells was performed by 1 hour incubation with the supernatant from dead cells. To prepare the supernatant, 2×10^6 EL4 cells were cultivated for 16 hours at 37°C and 7% CO₂ in 1 ml of HBSS. Antigen-coated wells were washed and incubated for 30 min with PBS-T solution (phosphate-buffered saline with 0.05% v/v of TWEEN® 20). 0.5 mg of the tested antibody in 100 ml of PBS-T were added into each well. The bound material was detected by adding peroxidase-labeled goat anti-mouse antibody (Cappel, Durham, NC) followed by the substrate. Absorbance in the visible spectrum at 405 nm was recorded using a Multiscan reader (Flow Labs., East Costa Mesa, CA) as an indication of relative reactivity in each well. Only the reactions giving an absorbance value at 405 nm, and which were five standard deviations above background absorbance, were scored as positive and marked with "+" (Table 1).

- 15 -

Table 1

Reactions of 2C5 With Intracellular Autoantigens in ELISA

| Antibody | Antigen | Dead EL4 cells | DNP | DNA | La | Ro | Sm | Jo-1 | Scl-70 |
|----------|---------|-------------------|-----|-----|----|----|----|------|--------|
| | | | | | | | | | |
| 2C5 | | + | + | - | - | - | - | - | - |
| UPC10 | | - | - | - | - | - | - | - | - |

As shown in Table 1, 2C5 bound only to the supernatant from dead EL4 cells and to DNP (Diamedix Corp.). Control antibody UPC10 did not bind to any of the antigens.

15 Anti-Tumor Effect of Autoantibodies

Tumors

A population of mammalian (preferably human or mouse) cancer cells (e.g., lymphoma, carcinoma, sarcoma, or myeloma) can be used to test the anti-tumor effect of an autoantibody such as an ANA from an aged mammal. The EL4 T cell lymphoma was selected for testing in C57BL mice because it is extremely aggressive, i.e., only about 10 tumor cells are sufficient to kill a mouse, and it is resistant to known methods of chemotherapy (Tarnovski, et al., Cancer Res., 39:3964-3967, 1979). In addition, mice do not have natural antibodies reactive against EL4 cells (Pierotti, et al., Int. J. Cancer, 18:223-229, 1976), and mice do not develop conventional antibodies against this virtually non-immunogenic tumor (Rees, et al., Chemotherapy, 28:283-290, 1982). Thus, this type of tumor is one of the most difficult to treat, and was selected for testing for this reason.

- 16 -

Tumor Inhibition In Vitro

The effect of a given autoantibody or ANA on the growth of a selected tumor cell, e.g., from a human patient, is tested *in vitro* using standard techniques well known in the art. For example, cells of a human cancerous tumor are extracted from the patient and cultured *in vitro* by standard techniques in the appropriate culture medium. The autoantibody, e.g., an ANA from an aged mammal (where the ANA is natural or monoclonal and the mammal is a mouse or human, for example) is contacted *in vitro* with the cultured human tumor cells in the presence of immune system effectors, e.g., murine or human, such as complement and/or cytotoxic cells, e.g., neutrophils and macrophages. Growth is determined, e.g., by monitoring the incorporation of derivatives of labelled exogenous thymidine, e.g., (methyl[³H] thymidine 15 Ci mmol⁻¹) (Amersham International, U.K.) into the DNA of treated and control tumor cell cultures. See, e.g., Curtin et al., Br. J. Cancer, 53:361-368 (1986). Standard cytotoxicity testing can be used to determine the percentage of dead cells in the culture. For example, the selective release of labelled chromium (e.g., Na₂⁵¹CrO₄, Amersham, Arlington Heights, IL) from dead cells into the culture medium can be monitored.

Tumor Inhibition In Vivo

In vivo testing of the effect of a selected autoantibody on the growth of human or other tumor cells is performed in humans or, preferably in an immunosuppressed animal, e.g., a nude mouse, carrying a xenogeneic tumor which has proliferated from artificially implanted tumor cells, e.g., tumor cells from a human, a mouse, or other mammal. The autoantibody, e.g., an ANA from an aged mammal, is formulated in a physiologically

- 17 -

acceptable carrier, and injected into the mouse carrying the tumor and proliferation is monitored.

For example, the following nude mouse assay can be used to test the anti-tumor effect of a selected ANA on a particular human tumor. Male athymic nude BALB/c mice (21-24 g) (Life Sciences, St. Petersburg, FL) are housed in a pathogen-free, temperature-controlled isolation compound and exposed to a light-controlled day (lights on, 0700-1900 h). Diet can be composed of standard chow (Autoclavable Rodent Chow No. 5010; Ralston Purina, St. Louis, MO) and water given ad libitum.

The tumor is xenografted as 2-mm² pieces s.c. through an interscapular incision bilaterally into the flanks (two tumors per mouse) of 12 anesthetized mice. Mice are weighed weekly, and tumors are measured twice weekly with calipers, and tumor surface areas calculated using the product of the two greatest perpendicular tumor diameters. Tumor doubling times are calculated from semilogarithmic graphs of tumor area versus days from implantation. At termination of the experiment, mice are sacrificed by cervical dislocation followed by excision of tumors and pancreas. Tissues are frozen in liquid nitrogen and stored at -70°C until assay.

Mice with implanted tumors are randomized and divided into two groups, each receiving 0.1 ml i.p. injections 3 times per week. The control mice receive saline and the mice in the treatment group receive the selected ANA in saline (e.g., 50 to 1000 µg, preferably 400 to 500 µg, per injection, per mouse).

The following test was carried out to demonstrate the anti-tumor effect of 2C5 on EL4 T cell lymphoma in mice. To simulate the natural situation of the aged mice, in which the neoplastic cells appear in a background of elevated serum ANA, experimental young mice were injected with 2C5 one day before (day -1) tumor cell

- 18 -

inoculation. Additional injections on days 1, 3, and 5 were used to maintain a sufficient level of the monoclonal antibody during early events of tumor development.

5 Experimental and control mice received a single inoculation of cultured EL4 lymphoma cells on day 0 (0.02×10^6 cells in 0.5 ml Hanks buffered salt solution (HBSS) per mouse). Antibodies formulated in a physiologically acceptable carrier were injected
10 intraperitoneally on days -1, 1, 3, and 5 (70 mg of purified antibody in 0.5 ml HBSS per mouse per injection). Mice were sacrificed by cervical dislocation on day 15 after the injection of 0.02×10^6 tumor cells, and on day 13 after the injection of 0.2×10^6 and 2×10^6
15 tumor cells. During the experiment animals were kept on a standard diet. Tumor size and appearance was recorded by photography, following which, subcutaneous tumors were cut out and weighed. Statistical analysis of relative tumor weight was performed to calculate "p" values (see,
20 e.g., Siegel, Nonparametric Statistics for the Behavioral Sciences, pp. 152-158. New York: McGraw-Hill Book Company, Inc., 1956).

Monoclonal antibody 2C5 purified from both ascites or sera-free hybridoma supernatant was effective in the
25 suppression of the development of the syngeneic EL4 lymphoma in C57BL/6 mice compared to control mice injected with isotype matched antibody produced by UPC10 myeloma (Cappel). The rate of subcutaneous tumor growth was substantially inhibited. Less than 25 per cent of
30 experimental mice injected with 20,000 EL4 lymphoma cells developed subcutaneously growing tumors after 15 days, compared with at least 75 per cent in controls (Figs. 1A and 1B and Table 2).

- 19 -

Table 2In Vivo Suppression of Tumor growth by Monoclonal 2C5

| | Number of EL4 cells injected | Treatment with | Number of mice with tumor | Tumor weight, g |
|----|--|-------------------|------------------------------|-------------------|
| 5 | 0.02x10 ⁶ | | | |
| | | HBSS | 13/17* | 1.58±0.64 |
| | | UPC10 | 5/5 | 1.56±0.17 |
| 10 | 0.2x10 ⁶ | 2C5 | 4/18** | 0.16±0.07 p<0.005 |
| | | HBSS | 3/4 | 1.30±0.58 |
| | | UPC10 | not done (ND) | ND |
| | | 2C5 | 2/5 | 0.42±0.29 p<0.1 |
| 15 | 2x10 ⁶ | | | |
| | | HBSS | 4/4 | 1.38±0.23 |
| | | UPC10 | ND | ND |
| | | 2C5 | 3/4 | 0.94±0.30 p<0.15 |
| 20 | *Cumulative data from three experiments, 5-6 mice in each. | | | |
| | **Cumulative data from four experiments, 4-6 mice in each. | | | |

The inhibition of tumor growth gradually decreased with the increase in the amount of lymphoma cells injected, from 0.02x10⁶ to 2x10⁶ cells per mouse (Table 2). These data evidence the prophylactic role of ANA from an aged animal in the control of natural tumor growth or development of metastases.

To further demonstrate the prophylactic effect of a selected autoantibody, it can be administered in a pharmaceutically acceptable carrier to normal animals at regular intervals to maintain a relatively constant level of the antibody in the blood. The animals are then exposed to a known carcinogen or specific amount of UV radiation that induces the development of cancer cells in control animals at a known rate. Similarly, known transgenic animals that are bred to develop tumors can be used to test the prophylactic effect of an autoantibody.

The binding characteristics of 2C5 described above provide evidence for two different potential mechanisms underlying the anti-tumor activity. First, flow cytometry data (Figs. 2A and 2B) demonstrated that 2C5 binds with the surface of living EL4 T lymphoma cells, but not normal T cells. In addition, 2C5 binds to the

- 20 -

surface of a variety of ther tumor cells such as S49 T lymphoma, P3X63-Ag8.653 myeloma, and VERO cells (green monkey kidney epithelium tumor cell line), which indicates that the antigenic determinant recognized by monoclonal antibody 2C5 is evolutionarily conserved (Klinman, J.Immunol., 148:1353-1358, 1992).

Different possible mechanisms for interaction of autoantibodies such as ANA with the cell surface have been described. Among those are the expression of a nuclear antigen on the cell surface (Prabhakar, et al., J. Clin. Invest., 86:1301-1305, 1990; Bachman, et al., Exp. Cell Res., 191:171-180, 1990), the possible cross-reactivity between nuclear and cell surface antigens (Lafer, et al., J. Exp. Med., 153:897-909, 1981), and binding of ANA to the cell surface as a part of an immunocomplex (Jacob, et al., Proc. Natl. Acad. Sci. USA, 86:4669-4673, 1989). Whatever the mechanism of interaction, the data obtained support a conclusion that 2C5, which belongs to the immuno-aggressive IgG2a isotype (Scholz, et al., Cancer Immunol. Immunother., 33:153-157, 1991), mediates antibody-dependent cellular cytotoxicity or/and complement-dependent lysis of tumor cells *in vivo*. Other isotypes such as IgG3 should also work. The class of IgG to which a given autoantibody belongs can be determined using standard techniques.

Second, 2C5 reacts with a protein released into the culture medium from dead tumor cells *in vitro* (Table 1). This finding supports another possible mechanism for the *in vivo* action of 2C5 on tumor development that can act concurrently with the complement-dependent lysis. It is known that, together with a large number of proliferating cells, every tumor contains some dead or dying cells (Wyllie, A.H., Anticancer Res., 5:131-136, 1985). Intracellular antigens released from the dead cells enter the circulation and create high local

- 21 -

concentrations in the tumor blood vessels.

Autoantibodies in the blood form immune complexes with some of those intracellular antigens, thereby elevating the local concentration of such complexes in the tumor vasculature. These complexes provide a local stimulation of the effector immune cells such as natural killer cells, neutrophils, and macrophages, which strongly inhibit further tumor development.

Use

- Existing tumors are treated in vivo by the administration (preferably by either intravenous or subcutaneous injection) of an anti-tumor autoantibody, e.g., an ANA of murine or human origin, formulated in a physiologically acceptable carrier. The proper dosage for therapeutic administration is determined by a physician for each case depending on the particular patient's age and weight, and on the size of the tumor. The dosage is within a range of 1 to 20 mg/kg of body weight of the patient to ensure that binding sites in the blood and on the surface of the tumor cells are saturated. Administration is continued until the tumor is destroyed, or used as an adjunct to other anti-tumor therapy or surgery until the tumor is destroyed or removed.
- Once a malignant tumor is located, the autoantibodies can also be used advantageously to prevent a secondary metastatic growth of the tumor. In this application, the antibodies are administered at periodic intervals to provide a relatively constant level of the antibodies in the blood stream until the original tumor is destroyed by the effect of the autoantibodies, or is removed surgically or by chemotherapy. The half-life of the autoantibodies in a mammal, e.g., a human, is on the average of several days, so a new dosage of

- 22 -

autoantibodies should be administered about once every 3 to 7 days, depending on the particular antibody. The specific regimen is based on standard blood tests that indicate the level of the autoantibody in the bloodstream. The dosage per injection is in the range of 1 to 20 mg/kg of body weight of the patient.

For other prophylactic uses, e.g., prior to the existence of a proliferating tumor, the dosage is again determined by a physician, but would be somewhat lower than the dosage required when a tumor is present, e.g., within a range of 0.1 to 5.0 mg/kg of body weight of the patient, depending on the patient's potential susceptibility to tumors based on, e.g., family history and DNA testing.

Human anti-tumor autoantibodies isolated from serum of healthy aged humans and administered directly, or in the form of human monoclonal antibodies derived from aged human cells, should have little, if any, toxic effects.

Deposit Statement

The hybridoma culture that produces monoclonal antibody 2C5 was deposited on June 22, 1994 with the A.T.C.C. in Rockville, Maryland under accession number ATCC CRL 11667. This culture is maintained under conditions that assure that access to the culture will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposit is available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the

- 23 -

subject invention in derogation of patent rights granted by governmental action.

Further, the subject culture deposit will be stored and made available to the public in accord with
5 the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., it will be stored with all the care necessary to keep the cells viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposits,
10 and in any case, for a period of at least 30 (thirty) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the culture plus five years after the last request for a sample from the deposit. The depositor acknowledges the duty to
15 replace the deposit should the depository be unable to furnish a sample when requested, due to the condition of the deposit. All restrictions on the availability to the public of the subject culture deposit will be irrevocably removed upon the granting of a patent disclosing them.

20

Other Embodiments

It is to be understood that while the invention is described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention,
25 which is defined by the scope of the appended claims. Other aspects, advantages, and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

For example, the anti-tumor autoantibodies of the
30 invention can also be used as tumor cell targeting agents that can be attached to known anti-tumor bioactive agents to augment the natural anti-tumor effect demonstrated for these autoantibodies themselves.

- 24 -

For example, the autoantibodies can be covalently linked to cytotoxins such as saponin, ricin, ricin A-chain; abrin, abrin A-chain, diphtheria toxin, diphtheria toxin A-chain, exotoxin A-chain, daunomycin, 5 daunorubicin, doxorubicin, mitomycin C, 5-fluorouracil, cytosine arabinoside, colchicine, cytochalasin B, bleomycin, vincristine, vinblastine, methotrexate, and other natural and synthetic, commercially available cytotoxins (Sigma Chemical Co., St. Louis, MO, for 10 example) known to those of ordinary skill in the art.

The autoantibodies also can be linked to cytokines such as interleukins, e.g., IL-2, interferons, e.g., gamma interferon, and growth factors, e.g., GM-CSF (Genzyme, Cambridge, MA).

15 The bioactive agent can be linked by a cleavable bond to the antibody if the toxin is internalized by the cancer cell to have an effect. For example the cleavable bond is enzymatically labile (such as a peptide linkage) for antibody-cytotoxin or antibody-cytokine complexes 20 that are internalized into the cell's cytoplasm or susceptible to hydrolysis by enzymes of the extracellular matrix. The bond may be pH labile for complexes that are phagocytized into the low pH environment of a lysosome.

The antibody is linked directly or indirectly to 25 the bioactive agent by a carrier molecule such as serum albumin (particularly human serum albumin), polyamino acids and dextran by methods well known to those of ordinary skill in the art. Convenient linkages include disulfides, imides, hydrazones, amides, and the like. 30 Multiple bioactive agent can be linked to the antibody. The multiple agents may be the same molecule or a mixture of agents linked to the antibody. The number of molecules linked to the antibody can vary depending on the size of the bioactive agent.

- 25 -

The autoantibody-cytotoxin or autoantibody-cytokine complex is administered to the mammal for the purpose of inhibiting tumor cell proliferation as described above for the antibody alone.

5 What is claimed is:

- 26 -

Claims

1. A composition for use in inhibiting the growth of a tumor cell in a mammal, said composition comprising an autoantibody that binds specifically to (i) a surface
5 of a living tumor cell and (ii) a protein released from a dead tumor cell or an antigen in the nucleus of said tumor cell, and (iii) does not bind to the surface of normal T cells.
2. The use of a composition for the manufacture
10 of a medicament for inhibiting the growth of a tumor cell in a mammal, said composition comprising an autoantibody that binds specifically to (i) a surface of a living tumor cell and (ii) a protein released from a dead tumor cell or an antigen in the nucleus of said tumor cell, and
15 (iii) does not bind to the surface of normal T cells.
3. A composition of claims 1 or 2, wherein said autoantibody is an antinuclear autoantibody.
4. A composition of claims 1 or 2, wherein said autoantibody is a substantially pure autoantibody
20 isolated from serum from an aged mammal.
5. A composition of claim 4, wherein said aged mammal is a human.
6. A composition of claim 4, wherein said aged mammal is a murine mammal.
- 25 7. A composition of claims 1 or 2, wherein said dead tumor cell protein is a deoxyribonucleoprotein.

- 27 -

8. A composition of claims 1 or 2, wherein said tumor cell is a human sarcoma, myeloma, carcinoma, or lymphoma cell.

9. A composition of claims 1 or 2, wherein said autoantibody is a monoclonal antibody produced by a hybridoma of a cell from an aged mammal.

10. A composition of claim 9, wherein said aged mammal is a murine mammal.

11. A composition of claim 9, wherein said aged mammal is a human.

12. Hybridoma cell line having A.T.C.C. accession number CRL 11667.

13. Monoclonal antibody 2C5 produced by the hybridoma cell line of claim 12.

14. A method of determining whether an autoantibody is capable of inhibiting growth of a tumor cell, said method comprising the steps of:

(a) obtaining a candidate autoantibody from a mammal;

(b) testing binding of said autoantibody to the surface of a tumor cell;

(c) testing binding of said autoantibody to the surface of a normal T cell; and

(d) testing binding of said autoantibody to a protein released from a dead tumor cell or an antigen in the nucleus of said tumor cell, wherein said autoantibody is capable of inhibiting growth of a tumor cell when said autoantibody tests positive in both of steps (b) and (d), and tests negative in step (c).

- 28 -

15. A method of claim 14, wher in said dead tumor cell protein is a deoxyribonucleoprotein.

1 / 3

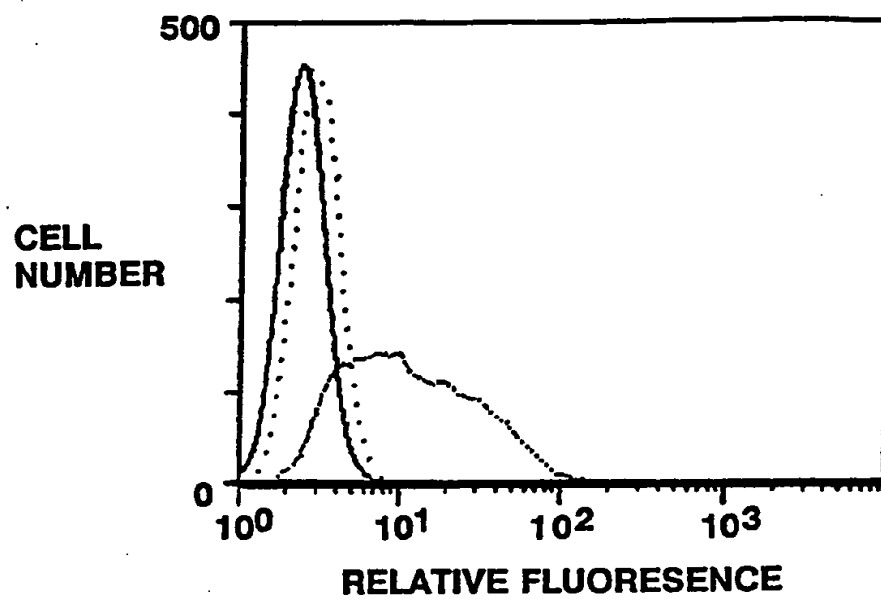
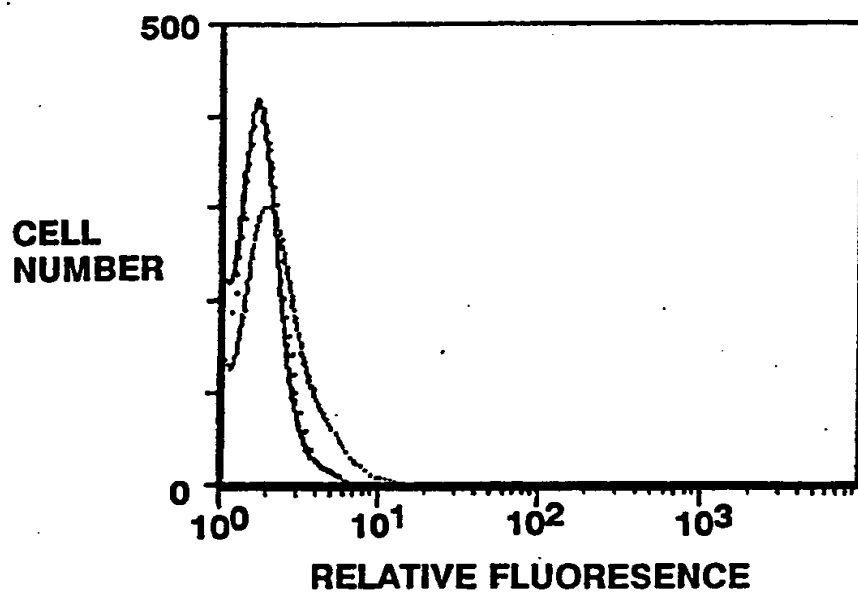


FIG. 1A

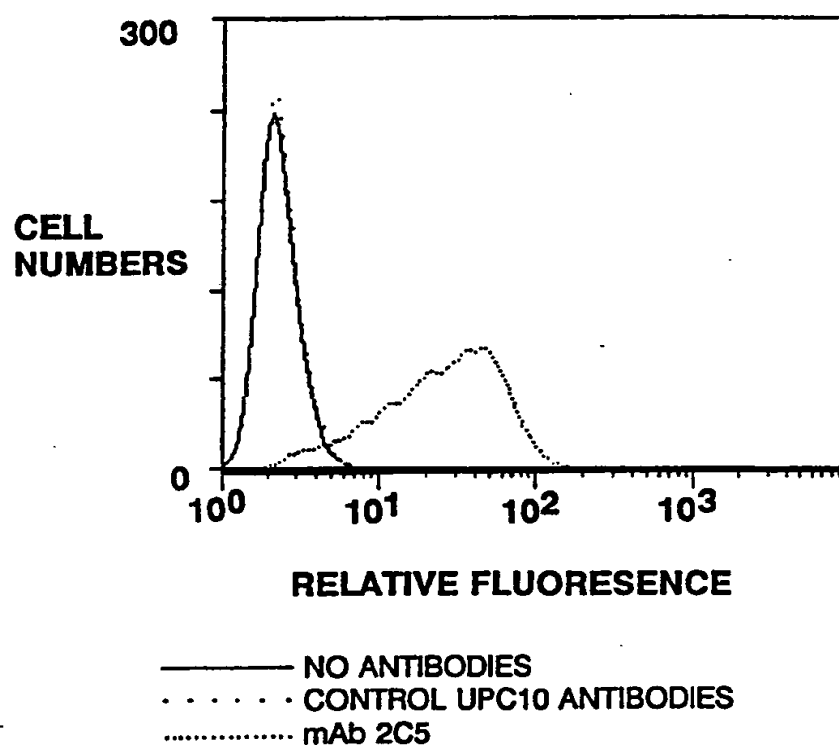


FIG. 1B

2 / 3

**FIG. 2A****FIG. 2B**

3/3

**FIG. 3**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07928

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/395; C07K 16/30; C12N 5/20, 5/24; C12P 21/08.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/136.1, 138.1, 155.1, 178.1; 435/70.21, 91, 240.27; 530/387.1, 388.21, 388.8.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| Y | IMMUNOLOGICAL INVESTIGATIONS, Vol. 22, No. 3, issued 1993, Banks et al., "Characterization of cross-reactive anti-DNA autoantibodies in murine lupus," pages 229-248, see entire document. | 1-15 |
| Y | IMMUNOLOGY, Vol. 61, issued 1987, Ricotti et al., "Autoantibodies to purified nuclear proteins related to DNA metabolism during ageing and in SLE patients," pages 375-381, see entire document. | 1-15 |



Further documents are listed in the continuation of Box C.



See patent family annex.

| | | |
|--|------|--|
| * Special categories of cited documents: | * T | Inter document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| * A* document defining the general state of the art which is not considered to be of particular relevance | * X* | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| * E* earlier document published on or after the international filing date | * Y* | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| * L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | * G* | document member of the same patent family |
| * O* document referring to an oral disclosure, use, exhibition or other means | | |
| * P* document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|--|
| Date of the actual completion of the international search 21 AUGUST 1995 | Date of mailing of the international search report 12 OCT 1995 |
| Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230 | Authorized officer <i>MR Woodhouse</i> ROBERT D. BUDENS Telephone No. (703) 308-0196 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07928

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| Y | JOURNAL OF THE NATIONAL CANCER INSTITUTE, Vol. 85, No. 24, issued 15 December 1993, Winter et al., "Antibodies against autologous tumor cell proteins in patients with small-cell lung cancer: Association with improved survival," pages 2012-2018, see entire document. | 1-15 |
| Y | J. W. GODING, "Monoclonal Antibodies: Principles and Practice," published 1986 by Academic Press (London), pages 59-77, 89-93, 125-133, 283-284, 289-290, see entire document. | 9-13 |
| Y | WO, A, 87/05031 (CENTER FOR MOLECULAR MEDICINE AND IMMUNOLOGY) 27 August 1987, see entire document. | 2, 9-13 |
| Y | BRITISH JOURNAL OF CANCER, Vol. 63, issued 1991, Langdon et al., "Characterization and properties of a small cell lung cancer cell line and xenograft WX322 with marked sensitivity to alpha-interferon," pages 909-915, see entire document. | 2, 9-13 |
| A | SCIENTIFIC AMERICAN, Vol. 271, No. 1, issued July 1994, Jain, R. K., "Barriers to Drug Delivery in Solid Tumors," pages 58-65, see entire document. | 1-15 |
| A | CANCER IMMUNOLOGY AND IMMUNOTHERAPY, Vol. 38, issued 1994, Chatterjee et al., "Idiotypic antibody immunotherapy of cancer," pages 75-82, see entire document. | 1-15 |
| A | BIO/TECHNOLOGY, Vol. 12, issued March 1994, Dermer, G. B., "Another anniversary for the war on cancer," page 320, see entire document. | 1-15 |
| A | IMMUNOLOGY TODAY, Vol. 11, No. 6, issued 1990, Osband et al., "Problems in the investigational study and clinical use of cancer immunotherapy," pages 193-195, see paragraph bridging pages 193-194. | 1-15 |
| A | ARTHRITIS AND RHEUMATISM, Vol. 36, No. 2, issued February 1993, Cronstein et al., "The adhesion molecules of inflammation," pages 147-157, see entire document. | 1-15 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/07928

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/136.1, 138.1, 155.1, 178.1; 435/70.21, 91, 240.27; 530/387.1, 388.21, 388.8.